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Original article

Opposite regulation of clusterin and LH receptor in the swine corpus luteum during luteolysis

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Abstract — Luteolysis, which occurs in a cyclical way to remove luteal tissue, may be an example of physiological apoptosis which counterbalances rapid tissue growth after ovulation. Clusterin is a multifunctional glycoprotein expressed in different tissues undergoing apoptosis. In this study we investigated clusterin and LH receptor gene expression during luteolysis as potential regulators of tissue growth and regression. Luteolysis was induced in pregnant sows (45 days) by Cloprostenol ($PGF_{2\alpha}$ analogue) treatment. Clusterin expression increased in the corpora lutea of pregnant sows ovariectomized 0, 6, 12, 24, 48 or 72 ($n = 3$) h after the luteolytic stimulus; maximum values were observed 24–48 h after the treatment ($P < 0.01$). An opposite trend between clusterin mRNA expression and markers of luteal function, such as progesterone levels in the corpora lutea and plasma, and LHr mRNA expression levels, was observed; moreover, clusterin expression was positively correlated with the degree of genomic DNA fragmentation, a marker of occurring apoptosis ($P < 0.01$). This pattern may be important in regulating luteolysis by a switch between luteotrophic and apoptotic stimulus. Our data indicate that P4 levels decrease prior to the increase in clusterin mRNA and the drop in LHr mRNA expression; we may therefore hypothesize a split between functional and structural luteolysis as reported in other species.

LH receptor / tissue growth / clusterin / apoptosis / luteolysis

1. INTRODUCTION

Clusterin (CLI) is a widely expressed glycoprotein with different putative functions. It has been demonstrated to inhibit the complement-mediated cytolysis [1] and to exert a protective influence on membrane function and lipid management [2] as well as a chaperone-like activity [3]. In addition, its expression appears to be associated with programmed cell death (apoptosis) [4] in a variety of organs and species: mouse mammary gland at weaning [5], rat prostate after castration [6], sex-hormone-dependent prostatic tumors after hormone withdrawal in humans [7], human kidney in response to

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ischemic injury [8], and rat thymocytes stimulated by dexamethasone [9]. Recently, the treatment of rat granulosa cells with CLI antisense oligonucleotide has been shown to result in an increase in apoptotic cell death, suggesting a functional protective role [10]. The structural luteolysis in different domestic species is accompanied by an active cell death [11–13]. In the swine, for example, luteal regression is dependent on a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-uterine release starting at day 13 post-ovulation [14]; prostaglandins are likely effective also through the stimulation of apoptosis, being that i.m. administration of Cloprostenol (a $PGF_{2\alpha}$ analogue) activates programmed cell death in porcine CL, with a genomic DNA ladder clearly evident after 24 h [13]. Apoptosis can also be induced by withdrawal of trophic factors. The main trophic factor of CL, Luteinizing Hormone (LH), exerts different effects on luteal tissue, including the stimulation of steroidogenesis and cell differentiation [14]. The effects of LH on luteal cells are mediated by the LH receptor (LHr), that has been observed to be expressed and steady-state regulated during the estrous cycle, pregnancy and luteolysis [14–16].

The purposes of this study were: (1) to determine the level of clusterin and LHr expression in the CL of pregnant sows throughout the Cloprostenol-induced luteolysis, and (2) to evidence the relationship between these parameters and both structural and functional luteolysis.

2. MATERIALS AND METHODS

2.1. Animals

Twenty-one prepubertal gilts (Large White × Landrace; 70–80 Kg on average) were artificially inseminated after hormonal treatments to induce estrus [13]; pregnancy diagnosis was performed after 30 d by ultrasound. Eighteen animals became pregnant (pregnancy rate 85.7%) and were used for experimental purposes. At 45 d of pregnancy all sows were i.m. injected with a single dose (75 $\mu g \cdot sow^{-1}$) of Cloprostenol (Dalmazin, Fatro, Italy). The animals were randomly assigned to six groups (3 sows per group) and ovariectomized (OVX) by aseptic laparotomy 0, 6, 12, 24, 48 or 72 h after Cloprostenol treatment. CL were isolated from the ovaries, weighed, counted and processed singularly. Each CL was dissected into two parts: one was used for total RNA extraction and the other was utilized for the biochemical evaluation of apoptosis and P4 tissue determination.

In order to check the efficiency of Cloprostenol treatment, blood samples were collected from the auricular vein before treatment (0 h) and at OVX time to determine P4 plasma levels.

All animals were housed and used according to EEC animal care guidelines. The experimental procedures had previously been submitted to and approved by the ethical committee of the Bologna University.

2.2. RNA isolation and RT-PCR

Total RNA was extracted by means of an RNA-fast isolation Kit (Molecular Systems, San Diego, CA, USA) from 5 corpora lutea/sow. Oligonucleotide primers were selected utilizing “Oligo” software (Med Probe, Oslo, Norway) and synthesized by Pharmacia Biotech (Milan, Italy). Primers for porcine clusterin were: 5’-AAG GAT CCC GAC CGC CAG CAG AGC CAC G-3’ (pCLISS-1) and 5’-TTG GAT CCG CCC CCC ATC CTT AGA AAC G-3’ (pCLISS-2). Primers for porcine LHr were: 5’-TAT TGA GCC TGG AGC ATT TA-3’ (LHrSS-1) and 5’-TGG AGT GTC TTG GGT GAG CA-3’ (LHrSS-2). Primers for porcine β-actin were: 5’-ATC GTG CGG GAC ATC AAG GA-3’ (ActSS-1) and 5’-AGG AAG GAG GGC TGG AAG AG-3’ (ActSS-2). The last eight nucleotides of pCLISS-1 and pCLISS-2 contained the BamHI sites suitable for cloning.

RT-PCR was performed with an Omni Gene (HYBAID, UK); all the chemicals were from Promega (Madison, WI, USA). For each PCR reaction, validation steps (optimization of calcium and magnesium concentrations and annealing temperature) were carried out as well as the examination of cycle number to look for the linear portion of the amplification curve. Equal amounts of total RNA from each sample (0.5 μg) were reverse transcribed at 42 ºC in a 20 μL reaction volume containing 1 U of RNasin, 2.5 U of AMV-RT (Avian Myeloblastosis Reverse Transcriptase), and 50 mM KCl, 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 1 mM deoxyribonucleoside triphosphates (dNTP), 2.5 μM random primers. Ten microliters of this mixture were used in each PCR reaction which contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM dNTPs, 7 μM of primers pCLISS-1/2 or LHrSS-1/2, and 0.5 μM of primers ActSS-1/2 in a final volume of 50 μL. The mixture was heated to 95 ºC for 10 min, cooled to 80 ºC and added with Taq polymerase (Promega) to a final concentration of 10 $U \cdot mL^{-1}$. The amplification of clusterin was performed utilizing a "touchdown" protocol [17] (2 cycles, 95 ºC for 1 min, 65 ºC for 1 min, 72 ºC for 1 min; 2 cycles 95 ºC for 1 min, 63 ºC for 1 min, 72 ºC for 1 min; 2 cycles 95 ºC for 1 min, 61 ºC for 1 min, 72 ºC for 1 min; 2 cycles 95 ºC for 1 min, 59 ºC for 1 min, 72 ºC for 1 min; 2 cycles 95 ºC for 1 min, 57 ºC for 1 min, 72 ºC for 1 min; 15 cycles 95 ºC for 1 min, 55 ºC for 1 min, 72 ºC for 1 min) then subjected to further extension at 72 ºC for 5 min, followed by incubation at 4 ºC. The amplification of LHr was obtained in 25 cycles with an annealing temperature at 50 ºC. The products of the reaction were separated on a Ethidium Bromide (EtBr)-stained 1.5% agarose gel and quantified by densitometric scanning (FluorS Max, Bio-Rad, CA, USA). Amplifications were carried out also on samples in which either RNA or reverse transcriptase was omitted from the RT mixture. The integrity of the RNA samples was tested by the coamplification of β-actin.

LHr mRNA was quantified relative to the β-actin amplicon obtained from each sample.

2.3. Competitive PCR

The target clusterin band (880 bp in length) was cut out from the agarose gel and eluted using GeneClean (Bio 101, Vista, CA, USA). The DNA was digested with

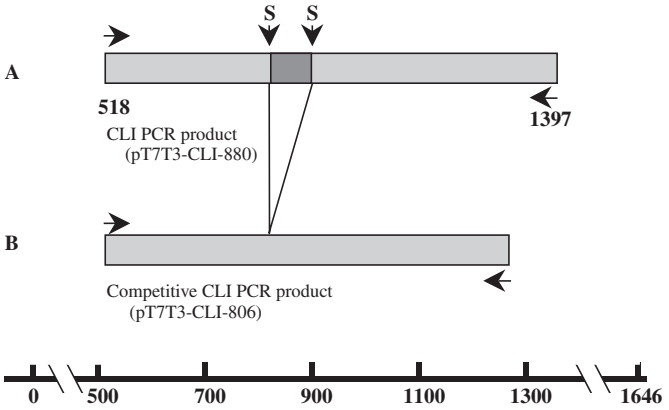

Figure 1. Porcine clusterin-PCR products. (**A**): Scheme of cloned normal CLI-PCR product (pT7T3-CLI-880) and Sau I (S) restriction endonuclease sites. (**B**): Scheme of subcloned competitive CLI (pT7T3-CLI-806), created by deleting a 74 bp Sau I internal fragment of A.

Bam HI at the recognition site level inserted at each 5' end of PCR primers, then ligated into the Bam HI site of the pT7T3-18U plasmid (Pharmacia, Uppsala, Sweden) to create pT7T3-pCLI-880 (Fig. 1A). *E. coli* NM522 competent cells were transformed by the recombinant plasmid [18] and the product was sequenced from both sides (MWG-AG Biotech Sequencing Service Ebersberg, Germany). A second plasmid (pT7T3-pCLI-806) was finally subcloned by deleting one Sau I internal fragment of 74 bp, from the pT7T3-pCLI-880 to discriminate native PCR products by molecular size (Fig. 1B).

Competitive PCR [19] was performed; known amounts of the 806 bp competitive cDNA, (5-10-50-100 fmol), were coamplified with the product of reverse transcription of total RNA isolated from porcine CL utilizing the same primers (pCLISS-1 and pCLISS-2). The amplification was performed as described above, without the addition of β-actin primers.

Reaction products were electrophoresed on 2% Amplisize agarose gel (BioRad, CA, USA) and quantitative analysis of the templates was performed by densitometric scanning of EtBr-stained gels. The ratio between the two products was calculated and the cDNA fmol value giving a 1:1 ratio was extrapolated. To correct for the difference in molecular weight, densitometric readings for target cDNA were multiplied 806/880. The ratio of the two amplicons was quantified for each dilution of competitive cDNA and the initial amount of clusterin cDNA was determined for each RNA sample.

2.4. DNA oligonucleosome detection

Low molecular weight DNA was isolated as previously described [13]. DNA (50 μg·lane^{-1}) was separated according to size by electrophoresis in a 2% EtBr agarose gel and quantified by densitometry; oligonucleosome formation was expressed as a percentage of low molecular weight DNA densitometric units (DNA < 1.3 Kb) vs. total densitometric units in the lane.

2.5. P4 determination

Plasma – Heparinized blood samples were centrifuged for 15 min at 800 *g*, and the plasma thus obtained was stored at –20 °C until the assay.

Luteal tissue – The tissues were homogenized in PBS (1:10 wt:vol), extracted with two volumes of ethanol, dried out and diluted in PBS (1:5 000 vol:vol).

P4 determination in both plasma and tissue samples were performed in triplicate by a validated RIA as previously described [13]. The sensitivity and ED50 for the assay were 4.6 and 65.6 pg·mL^{-1}, respectively; the blank value was < 1 pg·mL^{-1}. The intra- and inter-assay coefficients of variation were 11.2 and 13.9%, respectively.

2.6. Statistics

Data were analyzed using the Duncan new multiple range test after calculating a mean value for each sow (mean of five values for mRNA and mean of all the values for CL weight, P4 concentration and oligonucleosome detection). Each clusterin amplicon was normalized to the corresponding β-actin intratube amplicon [β-actin levels were unaffected by the treatment, $P > 0.45$ by one-way analysis of variance (ANOVA)]. The comparison of DNA ladder and mRNA levels was performed with the Pearson Correction Coefficient. The results are presented as means ± SEM.

3. RESULTS

3.1. Clusterin and LHr mRNA levels in pig CL during luteolysis

The clusterin 880 bp amplification product was clearly evident in all samples, as well as the LHr amplification product,

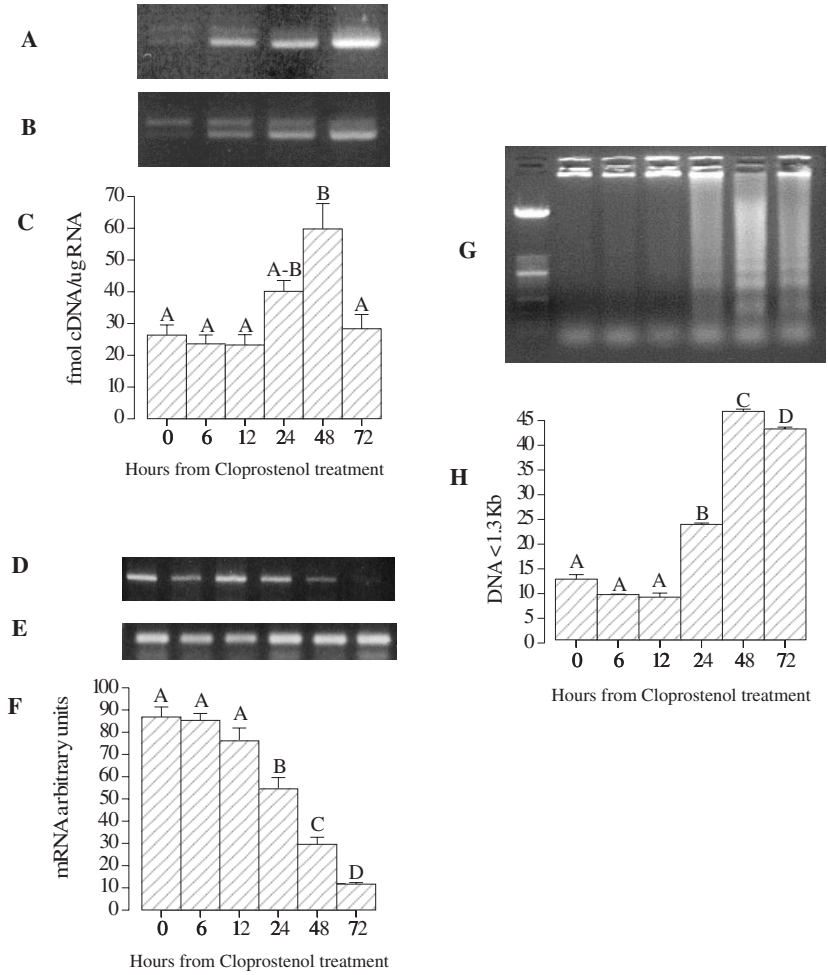

Figure 2. Expression of mRNA for clusterin (CLI) and LH receptor (LHr) and apoptosis determination in corpora lutea (CL) from pregnant sows ($n = 3$ per group) treated with a $PGF_{2\alpha}$ analogue. (**A**) Gel electrophoresis (2%) of competitive PCR amplification of CLI in a 0 h sample. (**B**) Gel electrophoresis (2%) of competitive PCR amplification of CLI in a 48 h after Cloprostenol administration sample. In A and B each lane represents a sample amplified together with dilutions of competitive DNA (5, 10, 50 and 100 fmol). (**C**) Quantification of CLI mRNA (fmol of cDNA·µg^{-1} of total RNA). The levels are reported as means ± S.E.M. Means with different superscripts are significantly different ($P < 0.01$) by the Duncan new multiple range test. (**D**) Gel electrophoresis (2%) of semi-quantitative RT-PCR of the LHr gene. (**E**) Gel electrophoresis (2%) of βActin RT-PCR. In D and E each lane is a representative amplification of time 0, 6, 12, 24, 48 and 72 h from cloprostenol treatment. (**F**) Quantification of LHr mRNA (arbitrary units); LHr mRNA products were normalized with the corresponding βActin. Levels are reported as means ± SEM. Means with different superscripts are significantly different ($P < 0.01$). (**G**) Gel electrophoresis (2%) of low molecular weight DNA. First lane = DNA molecular weight marker, subsequent lanes: 0, 6, 12, 24, 48, 72 h samples. (**H**) Quantitative analysis of low molecular weight DNA (< 1.3 Kb). Each data point represents the mean ± SEM of three independent DNA extractions and gel runs. Bars with different letters are significantly different ($P < 0.01$).

Table I. Corpora lutea weight and P4 levels in plasma and luteal tissue of pregnant sows treated with a $PGF_{2\alpha}$ analogue (means ± SEM). Different letters in the same line indicate significant ($P < 0.01$) difference.

Parameters	Time after Cloprostenol treatment (h)					
	0 ($n = 3$)	6 ($n = 3$)	12 ($n = 3$)	24 ($n = 3$)	48 ($n = 3$)	72 ($n = 3$)
CL weight (g)	0.57 ± 0.07^{A}	0.53 ± 0.05^{A}	0.44 ± 0.04^{A}	0.23 ± 0.01^{B}	0.17 ± 0.04^{B}	0.19 ± 0.02^{B}
Tissue P4 ($ng \cdot mg^{-1}$)	82.53 ± 1.76^{A}	22.59 ± 4.39^{B}	26.33 ± 0.77^{B}	10.4 ± 0.51^{C}	9.43 ± 1.40^{C}	4.8 ± 0.25^{C}
Plasma P4 ($ng \cdot mL^{-1}$)	19.28 ± 1.47^{A}	4.83 ± 0.39^{B}	2.78 ± 0.11^{C}	1.86 ± 0.37^{C}	0.93 ± 0.14^{C}	0.72 ± 0.20^{C}

687 bp in length. Pig β-actin, 169 bp, was also co-amplified showing that all RNA were intact. The sequence of the cloned clusterin PCR product confirmed the identity with the pCLI mRNA expressed in pig cultured aortic smooth muscle cells (nucleotides 518–1 397) [20].

Clusterin mRNA levels averaged 24.36 fmol cDNA·μg^{-1} RNA during the first 12 h of the experimental period, then increased reaching the maximum value 48 h after the luteolytic stimulus ($P < 0.01$); after that it returned to their initial values (Figs. 2A, 2B and 2C). LHr mRNA was high during the first 6 h, then it progressively decreased to the lowest value (Figs. 2D, 2E and 2F).

3.2. Biochemical evidence of apoptosis

Levels of nucleosomal DNA fragmentation were low during the first 12 h after Cloprostenol, then significantly ($P < 0.05$) increased with maximal values at 48 h (Figs. 2G and 2H). Low molecular weight DNA levels were correlated positively with clusterin mRNA values and negatively with LHr mRNA ones (Pearson correlation coefficient = $P < 0.01$; $r = 0.59$ and 0.86, respectively).

3.3. Corpora lutea weight and P4 levels

The average number of CL/sow was 21.42 ± 2.56 (mean ± SEM) without any significant difference among groups. Data on CL weight and P4 concentrations in both plasma and luteal tissue are reported in Table I. The mean weight of CL collected during the first 12 h was significantly ($P < 0.05$) different from that of CL recovered during the subsequent period. P4 levels in both plasma and luteal tissue started to decrease in a similar way, immediately after Cloprostenol treatment, with a significant difference between 0 and 6 hours ($P < 0.01$); after that P4 concentrations continued to decrease, reaching the lowest values by the end of the experiment.

4. DISCUSSION

The cDNA of porcine clusterin was cloned from vascular smooth muscle cells [20] and the protein encoded by this cDNA shows a 72% sequence homology with human CLI. Clusterin is related to apoptosis in several tissues from different species [21], although its expression has not been demonstrated in some tissues [22]; therefore, the relationship between CLI expression and the activation of the apoptotic program is not completely clear so far, being that CLI expression has also been reported to be involved in cell survival rather than in cell death [23].

Data on CLI in the female reproductive tract are scarce. Clusterin has been demonstrated in both human and rat ovaries [24–25]; in the rat, CLI expression decreases during follicular growth and is related to luteolysis [26].

We have previously shown that apoptosis is involved in $PGF_{2\alpha}$-induced regression of porcine CL; apoptosis is evident as early as 6 h after $PGF_{2\alpha}$ administration by the 3'OH in situ technique, and after 24–48 h by DNA ladder determination [13].

One of the objectives of this study was to investigate whether CLI-mRNA is expressed in swine CL and if its expression is related to the apoptotic phenomenon that occurs during $PGF_{2\alpha}$-induced structural regression of CL. The results from this study demonstrate that CLI is expressed in functional corpora lutea of pregnant sows, as well as during luteolysis, and that its expression increases as DNA fragmentation augments; similar data have been reported in rats [26]. Previous results from our laboratory indicate that signs of apoptosis in CL stromal tissues (endothelial) occur very early (6 h) [13], prior to any modification in CLI expression (data from this experiment); as for the morphological aspects, luteal cells do not present any evidence of change up to 24–48 h after the luteolytic stimulus [13]. These results suggest that in pig CL, clusterin may be involved in the mechanisms of membrane remodeling that occurs during the advanced stages of luteolysis rather than in the early ones; clusterin may also be involved in modulating lipid metabolism. Steroidogenesis, the predominant process in luteal cells, is dependent on cholesterol, mainly derived from circulation, as the initial substrate; luteal cells contain LDL and/or HDL receptors (at least in part LH-regulated [27]) that are involved in lipoprotein incorporation into the cell. CLI plays a key role in the biochemical machinery responsible for cholesterol turnover [23, 28]; therefore it is not surprising that it is expressed in the functional CL as in other tissues in which lipid metabolism is fundamental (brain and liver) [20]. During luteal cell involution, in fact, many lipid droplets and cytoplasmic vacuoles as well as a 10-fold increase in luteal triglycerides are observed; the antisteroidogenic effect of $PGF_{2\alpha}$ appears to be mediated through the PKC inhibition of cholesterol transport to cytochrome P450scc [29]. CLI upregulation has been shown to be induced by phosphatidylserine-containing lipid vesicles [30]. The "scavenger activity" of CLI during tissue repair [31] and its ability to form a high density lipoprotein complex with apolipoprotein A-1 [32] can support the hypothesis of an involvement of CLI in the reverse cholesterol transport, that allows its removal from cytoplasm and its redistribution [28, 32], thus contributing to the shortage of the substrate for steroid production.

As for LHr, its expression changes throughout the estrous cycle and pregnancy in both bovine and swine species [15, 16, 33, 34]; LHr mRNA is very low in regressing CL as compared to mid-cycle CL or during pregnancy. LH receptors in swine and bovine corpora lutea have been reported to decrease immediately after $PGF_{2\alpha}$ administration [35, 36] and continue to decline during the following 24 h; a similar trend has been reported, at least in swine, for both luteal and serum progesterone concentrations, so that all these parameters present a similar profile. The results from this study, on the contrary, indicate that both plasma and luteal P4 concentrations diminish before the reduction of LHr mRNA (which seems to be more related to the reduction in CL weight); these data are in agreement with those by Grinwich et al. [37], who observed that the drop in luteal LH receptors in $PGF_{2\alpha}$-treated rats is less pronounced than that of progesterone concentrations. Also Spicer et al. [38] in the cow, Diekman et al. [39] in the sheep and Roser et al. [40] in the mare reported that the drop in blood progesterone levels precedes by several hours the drop in LH receptors or hCG binding sites in CL from $PGF_{2\alpha}$-injected animals.

As suggested [38], the decrease in LH receptors does not seem to be the first step in corpora lutea regression which is likely

associated to other events such as a direct antagonism of LH by $PGF_{2\alpha}$ [37]. These authors hypothesized that the very rapid effect of prostaglandins on progesterone secretion probably occurs through a reduction in the LH-induced hyperemic activity at the ovarian level; the loss of LH receptors possibly ensures the subsequent progression of the luteolytic process. Tissue growth, homeostasis and regression could be the final result of the activity of several factors that, at the same time, exert opposite effects. As mentioned above, the switch between the persistence of a luteotrophic stimulation (LHr expression) and a prevalent luteolytic activity (as timed by clusterin expression) is possibly located 24 h after Cloprostenol stimulation.

Collectively, our findings indicate that both clusterin and LHr mRNA are regulated during the luteolytic process. However, being that P4 levels markedly decrease prior to significant changes of both mRNA levels, we may hypothesize that these genes might be involved in the later stage of structural regression rather than in the induction of cell death; the decline of LHr leads to a block in cellular division while the CLI rise protects the surviving cells during the apoptotic process and acts as a regulator of lipid transport and redistribution.

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